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Identification of a Novel Variant of Hepatitis E Virus in Italy

Alessandro R. Zanetti,[1]* George G. Schlauder,[2] Luisa Romanò,[1] Elisabetta Tanzi,[1] Paolo Fabris,[3] George J. Dawson,[2] and Isa K. Mushahwar[2]

[1]*Institute of Virology, University of Milan, Milan, Italy*
[2]*Viral Discovery Group, Abbott Laboratories, North Chicago, Illinois*
[3]*Department of Infectious Diseases, S. Bortolo Hospital, Vicenza, Italy*

Hepatitis E infection is typically associated with areas in which hepatitis E virus (HEV) is endemic. Except for a few cases in Europe and in the United States, acute hepatitis E is usually associated with travel to endemic areas. We set out to determine the etiologic role of HEV in acute non-A-C hepatitis in Italy. The presence of HEV-RNA and antibody was determined in 218 patients diagnosed with acute viral non-A-C hepatitis. Acute hepatitis E infection was defined by the presence of HEV-RNA in sera and positivity for IgM anti-HEV and seroconversion to IgG anti-HEV. Acute hepatitis E was found in 10.1% of the patients with acute non-A-C, with 95.5% exhibiting a benign course. A more severe course was observed in a patient co-infected with HAV and HEV. Most cases were travelers to endemic areas, although 18.2% reported no travel. One patient was from a household with an infected patient. Sequence analyses of the polymerase chain reaction (PCR) product derived from a patient who never visited endemic areas, identified an isolate that is divergent significantly from all reported isolates of HEV (79.5–85.8% nucleotide identity). Evidence from this study suggests that HEV accounts for approximately 10% of acute non-A-C viral hepatitis in Italy, diagnosed generally in travelers returning from endemic areas. However, the identification of a new HEV variant in an individual who never indicated travel or contact with individuals associated with endemic areas, suggests that this virus may be native to Italy. ***J. Med. Virol. 57:356–360, 1999.***

KEY WORDS: hepatitis E; HEV variant; non-A-C hepatitis

INTRODUCTION

Viral hepatitis type E is a major public health problem in the developing world, where the infection occurs both sporadically and in large outbreaks [Purcell and Ticehurst, 1988; Balayan, 1997]. In Western Europe and in the United States (US), the disease is diagnosed occasionally in travelers from endemic areas, but sporadic cases have also been identified in patients who never visited endemic countries [Bader et al., 1991; Dawson et al., 1992; Centers for Disease Control and Prevention, 1993; Zaaijer et al., 1993; Skidmore and Sherrat, 1996). Serological surveys carried out in industrialized countries have shown that the prevalence of anti-hepatitis E virus antibody (anti-HEV) is 1–6% among blood donors and much higher in some risk groups and patients with liver disease other than acute viral hepatitis [Halfon et al., 1994; Montella et al., 1994; Paul et al., 1994; Pisanti et al., 1994; Zanetti et al., 1994; Buffet et al., 1996; Romanò et al., 1996]. The meaning of the relatively high rate of anti-HEV compared with the very low rate of clinically overt hepatitis E in these nonendemic areas is unclear. Inadequacy of the available serological assays, circulation of native viral variant(s) at low level of pathogenicity, or presence of other related viruses sharing antigens with HEV have been suggested as possible explanations for these findings.

Recently, a novel HEV isolate, significantly divergent from the original Mexican and Burmese strains, was identified in the US [Kwo et al., 1997; Schlauder et al., 1998] in a patient with acute hepatitis with no history of travel to areas where HEV is endemic. This finding provided evidence that HEV is also present in countries considered previously to be free of any native strains and lends support to the notion that HEV variants may be circulating in geographical areas not previously considered to be HEV endemic.

The aim of this study was to determine the etiological role of HEV in acute non-A-C hepatitis in Italy, and

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*Correspondence to: Prof. A.R. Zanetti, Institute of Virology, Medical School, University of Milan, Via C. Pascal 38, 20133 Milan, Italy. E-mail: zanetti@imiucca.csi.unimi.it

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to analyze the nucleotide and derived amino acid sequences of an isolate from a patient with no history of travel to endemic areas and to compare it with the reported sequences of other HEV strains.

PATIENTS AND METHODS

Patients

Between January 1994 and December 1997, 218 patients (123 males and 95 females; mean age 37.5 years, range 1–79 years) attending several National Infectious Diseases Units, were diagnosed with acute viral non-A-C hepatitis (negative for IgM anti-HAV, HBsAg, IgM anti-HBc, anti-HCV, HCV-RNA, and exclusion of autoimmunity, alcohol, or hepatotoxic drugs). Serum samples obtained during the acute and convalescent phases of illness and stools collected from 28 (12.8%) patients were stored at –80°C for subsequent testing. Informed consent was obtained from each patient who entered in the study.

Hepatitis E was defined by the presence of HEV-RNA in sera or stools and positivity for IgM anti-HEV and seroconversion to IgG anti-HEV. To identify the risk factors for hepatitis during the 6 months preceding the onset of the illness, each patient was interviewed with a pre-coded questionnaire.

Laboratory Analysis

Laboratory stools and serum samples from the various consecutive bleeds were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, HEV-RNA was extracted from fecal suspensions (1 g feces in 10 ml of phosphate-buffered saline) and from sera by proteinase K digestion and phenol-chloroform extraction. Nucleic acid amplification by RT-PCR utilizing the GeneAmp RNA PCR Kit (Perkin-Elmer/Roche, Branchburg, NJ) was carried out using nested primers [Schlauder et al., 1993] derived from the open reading frame 1 (ORF1) of the viral genome of the original Burmese viral strain. Anti-HEV antibodies of IgG class were detected by an enzyme-linked immunosorbent assay system (EIA, Abbott Laboratories, North Chicago, IL), whereas those of IgM class were tested by an "in house" modified EIA. Briefly, the IgG antibodies were removed by chromatography of serum samples on a column containing recombinant protein G immobilized on Sepharose 4B. The IgG-depleted sera were then assayed by EIA using the same recombinant HEV antigens used in the IgG test as solid phase and goat anti-human IgM (μ specific) antibodies conjugated to horseradish peroxidase (Boehringer Mannheim GmbH, Mannheim, Germany) as probe. HBsAg, IgM anti-HBc, IgM anti-HAV, anti-HCV were tested with EIA kits available commercially (Abbott Laboratories, North Chicago, IL, USA), and HCV-RNA was assayed by RT-PCR with nested primers derived from the 5′- noncoding region of the viral genome [Puoti et al., 1992]. Liver function tests were carried out by routine methods.

Sequence analysis. Amplification for identification of the novel isolate from the only sample available from the four patients who did not indicate travel to endemic areas was performed as described above. PCR primers were based on the HEV US-1 isolate (sense primer, 5′-TGG CAT TAC TAC TGC CAT TG-3′, antisense primer 5′-AGG AAA CAC CGA TGC AGA AC-3′) and amplified a fragment from the 5′-end of ORF1 corresponding to nucleotides 57 through 350 of the Burmese sequence [Tam et al., 1991; Schlauder et al., 1998]. PCR was conducted using thermal cycling conditions known as "touchdown PCR," to enhance detection of mismatched primer-template pairs [Schlauder et al., 1998]. The PCR product was cloned into pT7-Blue T-Vector plasmid (Novagen, Abingdon, United Kingdom) and sequenced on a ABI model 373 DNA Sequencer using ABI Sequencing Ready Reaction Kit. Pairwise alignments of the nucleotide and amino acid sequences were performed using the program GAP of the Wisconsin Sequence Analysis Package (Version 8 and 9) to generate nucleotide identities and deduced amino acid identities and similarities. Sequences used in the analyses were the same as those in Schlauder et al. [1998]: Burmese 1 and 2, B1 and B2; Pakistan 1, P1; Chinese 1, 2, 3 and 4, C1, C2, C3 and C4; Indian 1 and 2, I1 and I2; Mexican 1, M1; United States 1, U1.

Statistical analysis was undertaken by Chi-square test for frequencies. Group means were compared with Student's *t*-test.

RESULTS

Twenty-two (19 males and 3 females; mean age 28.8 years, range 3–50 years) of 218 (10.1%) patients examined were diagnosed with acute hepatitis E (Table I). At the time of hospitalization, the alanine aminotransferase (ALT) mean peak was 2,720 IU/l (range 396–12,290 IU/l) and all patients' sera were positive for HEV-RNA and anti-HEV of both IgM and IgG class. Stools collected from 6 of these 22 patients were also found to be positive for HEV-RNA. Compared with patients with acute non-A-E hepatitis, those with acute hepatitis E were younger, more frequently males, and had more severe acute disease with higher ALT levels. As for risk factors, 17 HEV-positive patients (77.3%) developed acute hepatitis on their return from travel to endemic areas (8 patients from India, 3 from Pakistan, 5 from Bangladesh and 1 from Somalia). One patient (5 years old) acquired infection from her sister (3 years old) who had developed acute hepatitis E two days after her return from Somalia. Four patients (18.2%) denied travel abroad and contacts with people coming from endemic areas or other risk factors such as drug addiction or shellfish consumption.

The acute disease had a benign course with normal ALT within 3–5 weeks in 21 patients (95.5%); no clinical difference was seen between patients who acquired HEV abroad and those who never visited endemic areas. One patient (male, 50 years old) acquired HEV and HAV co-infection during a trip to India. This patient, who was found positive for HEV-RNA and anti-HEV of both IgM and IgG class and IgM anti-HAV negative at hospitalization, became IgM anti-HAV positive 1 week later. The clinical disease had a more severe course

TABLE I. Clinical and Demographic Characteristics of the Patients With Acute Type Non-A-E and E Hepatitis

	Acute hepatitis nA-E (n = 196)	Acute hepatitis E (n = 22)[a]	P
Age (years)			
Mean (±SD)	38.5 (±16.8)	28.8 (±12.8)	0.006
Median	38	29	
Range	1–79	3–50	
Sex			
M/F	104/92	19/3	0.005
ALT (IU/l)			
Mean (±SD)	1188 (±1143.8)	2720 (±2606.8)	0.02
Median	825	2071	
Range	101–4940	396–12290	
Risk factor			
Unknown	61.2%	18.2%	
Travel	12.8%	77.3%	
Secondary case	—	4.5%	
Shellfish	7.7%	—	
Hospitalization/Surgery/Dental care	5.5%	—	
Transfusion	3.6%	—	
Health care	3.6%	—	
IVDUs	3.1%	—	
Other[b]	2.5%	—	

SD, Standard Deviation.
[a]One case was HEV and HAV co-infected.
[b]Tattooing, piercing, etc.

(ALT peak 12,290 IU/l) than that observed among patients infected with HEV alone, with normalization of liver function tests within 8 weeks. The mean duration of HEV-RNA, calculated for the 22 patients with acute hepatitis E, was 8 days in sera (range 3–19 days) and 11 days in stools (range 8–15 days). Anti-HEV of IgM class tended to disappear in all patients within 3–4 weeks, whereas anti-HEV of IgG class continued to be detected at declining levels throughout the follow-up period (Fig. 1).

PCR primers based on sequence from a variant of HEV isolated recently in the US [Kwo et al., 1997] generated a product of the expected size, 294 base pairs, from a patient who had no history of travel to HEV-endemic areas. Sequencing of the PCR product permitted comparison of the 251 base pairs between the primer sequences with sequences from other HEV isolates. Sequence identities from pairwise alignments of the nucleotide and deduced amino acid sequences are shown in Table II. The comparisons of the nucleotide identities indicate that the Italian isolate, designated It-1, was significantly divergent from the original Mexican isolate (79.9%), as well as a number of Asian isolates that have been sequenced completely (79.5–81.1%). In addition, the Italian HEV isolate was also significantly divergent from the novel US isolate (85.8%).

DISCUSSION

All sera collected at the time of hospital admission from the 22 patients diagnosed with hepatitis E were HEV-RNA and IgM and IgG anti-HEV antibody positive. Stools collected from 6 of these 22 patients were also positive for HEV-RNA. Fecal shedding of HEV and viremia were found to occur predominantly during the first 10 days after the onset of acute hepatitis and were no longer detectable in samples collected 3 weeks after the onset of the disease. Similarly, the presence of IgM anti-HEV was short lived and cleared within 4 weeks, whereas antibody of IgG class was found to persist in all HEV-positive patients for the entire follow-up period (up to 25 months).

In all patients but one, hepatitis E had a benign course and no difference in the duration of clinical symptoms and in the mean ALT levels was seen between patients who traveled to endemic areas and those who did not. Interestingly, the patient who developed the more severe clinical hepatitis was co-infected with HAV and HEV. Whether the outcome of hepatitis E may be worsened when additional hepatotropic viruses infect the same person concurrently needs to be studied further.

Hepatitis type E is an enterically transmitted disease that is responsible for a majority of sporadic and epidemic cases of acute viral hepatitis in tropical and subtropical countries [Purcell and Ticehurst, 1988; Balayan, 1997]. The disease occurs primarily in young adults and is self-limited, but has a high mortality rate in pregnant women, particularly during the third trimester of pregnancy. In Europe, the diagnosis of acute hepatitis type E is unusual and related generally to travel in endemic areas. However, sporadic cases not associated with traveling to the tropics have been diagnosed in the US, the Netherlands, Greece, Spain, Turkey, and Italy [Zaaijer et al., 1993; Koksal et al., 1994; Psichogiou et al., 1995; Quiroga et al., 1996; Cacopardo et al., 1997; Kwo et al., 1997]. The presence, although at low level, of HEV in the European Mediterranean basin is not surprising, because this virus is endemic in neighboring countries such as those of North Africa and Middle East. In this context, the fre-

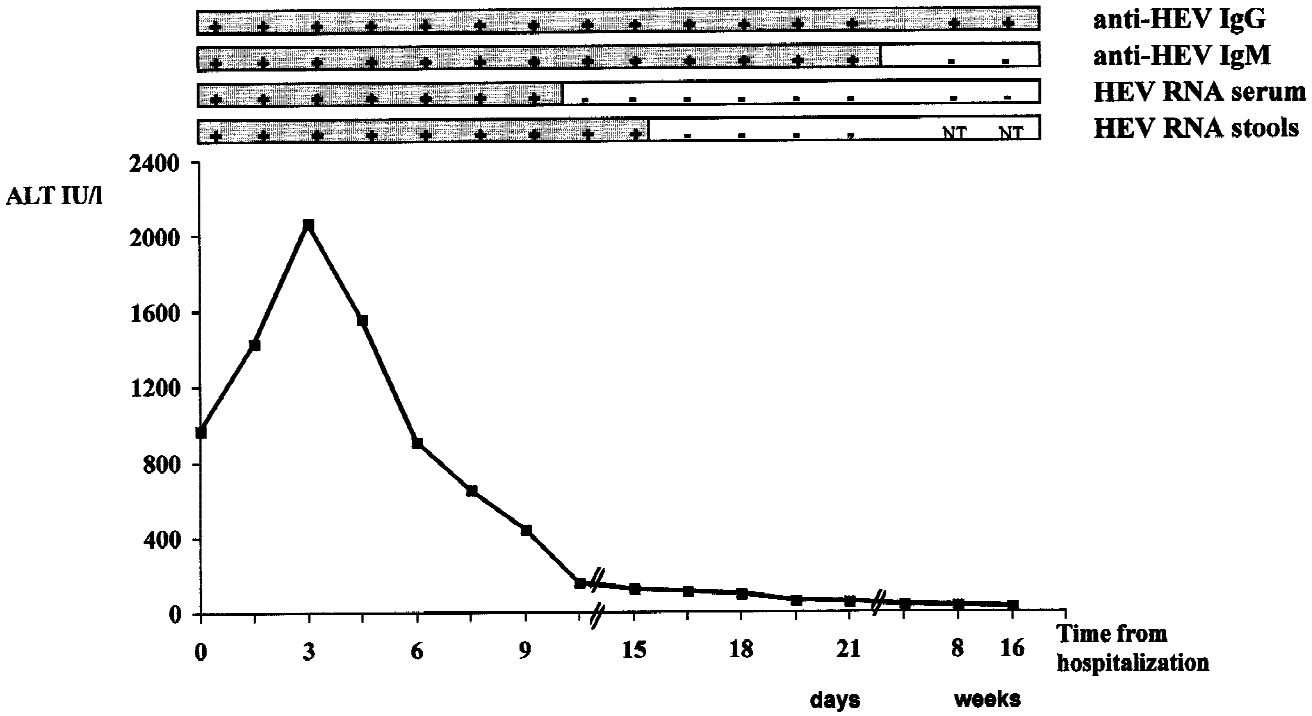

Fig. 1. Serological and virological features of a patient who developed acute hepatitis E after travelling to India. NT = not tested.

TABLE II. Nucleotide Identity and Deduced Amino Acid Identity/Similarity Between Isolates of HEV Over 252 Base Pair Fragment From ORF1

Nucleotide Identity											
B1	99.2	95.7	95.7	96.1	95.7	94.9	95.3	96.5	82.3	74.8	80.7
100/100	**B2**	94.9	94.9	95.3	94.9	94.1	94.5	95.7	81.5	75.2	80.3
100/100	100/100	**P1**	98.4	99.6	98.4	97.6	94.9	92.9	83.1	76.0	80.3
100/100	100/100	100/100	**C1**	98.8	98.4	96.9	94.1	92.9	83.8	76.4	80.7
100/100	100/100	100/100	100/100	**C2**	98.8	98.0	95.3	93.3	83.5	76.0	80.3
100/100	100/100	100/100	100/100	100/100	**C3**	96.8	94.1	92.9	83.1	76.0	80.3
100/100	100/100	100/100	100/100	100/100	100/100	**C4**	94.1	92.9	82.7	76.0	79.5
100/100	100/100	100/100	100/100	100/100	100/100	100/100	**I1**	92.5	81.9	76.8	81.1
98.8/98.8	98.8/98.8	98.8/98.8	98.8/98.8	98.8/98.8	98.8/98.8	98.8/98.8	98.8/98.8	**I2**	81.5	75.6	80.3
98.8/97.6	98.8/97.6	98.8/97.6	98.8/97.6	98.8/97.6	98.8/97.6	98.8/97.6	98.8/97.6	97.6/96.4	**M1**	77.6	79.9
92.9/91.7	92.9/91.7	92.9/91.7	92.9/91.7	92.9/91.7	92.9/91.7	92.9/91.7	92.9/91.7	91.7/90.5	94.0/92.9	**U1**	85.8
94.0/94.0	94.0/94.0	94.0/94.0	94.0/94.0	94.0/94.9	94.0/94.0	94.0/94.0	94.0/94.0	92.9/92.9	95.2/95.2	97.6/96.4	**It1**
Amino Acid Similarity/Identity											

Isolates represented are Burmese (B1, B2); Pakistan (P1); Chinese (C1, C2, C3, C4); Indian (I1, I2); Mexican (M1); United States (U1); Italian (It1).

quent commercial and cultural exchanges between these populations and, more recently, the impressive migratory flux from HEV-endemic areas to Europe has been considered as a possible cause for maintaining the circulation of the virus in these countries.

From our study it emerges that, in Italy, HEV accounts for approximately 10% of the acute non-A-C hepatitis. Most cases (18/22; 81.8%) were travelers to endemic areas, with 18.2% (4/22) reporting no travel or links with people coming from endemic areas in the 6 months preceding the onset of the disease. These cases of acute hepatitis E acquired domestically indicate that HEV may circulate in Italy. This finding is in agreement with the several seroepidemiological studies, which have shown that anti-HEV is detectable in the Italian population with a gradient of positivity between northern and southern regions [Cacopardo et al., 1997; Coppola and Masia, 1997]). In addition, the identification of a novel HEV variant stresses the importance of testing for genetic diversity of HEV circulating in different areas. Isolates of HEV have been described from a number of geographic locations, including Mexico, Burma, China, Pakistan, Africa, and the US [Tam et al., 1991; Huang et al., 1992; Tsarev et al., 1992; Yin et al., 1994; Chatterjee et al., 1997; van Cuyck-Gandrè et al., 1997; Schlauder et al., 1998]. The Italian isolate appears to be related most closely to the US isolate with a nucleotide identity of 85.8%. Although this percentage is greater than the range of identities compared with the Mexican and other Asian strains (79.5–

81.1%), the identity is still less than that observed for the most distant Asian isolates, I1 and I2, at 92.5%. The identification of a distinct variant from a patient in Italy, who developed acute hepatitis E with no history of travel to endemic areas, is suggestive for the circulation of an endogenous HEV in Italy. The sensitivity and specificity of assays for anti-HEV antibody are important factors in assessing the worldwide distribution of HEV infection. A recent study on the evaluation of assays for anti-HEV showed that the detection of antibody by different tests varied by the geographical origin of the inoculum, probably due to differences in the geographic strain-specific antigenic domains included in these tests [Mast et al., 1997]. The cloning, sequencing, and preparation of synthetic or recombinant peptides based on immunogenic regions of this novel HEV variant may lead to the design of specific enzyme immunoassays useful for understanding the epidemiology of HEV infection in Europe.

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